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# Characterization of Neuraminidases Produced by Various Serotypes of *Pasteurella multocida*

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**Neuraminidases produced by 16 strains of *Pasteurella multocida* (serotypes 1 to 16) were characterized by molecular weight, substrate specificity, and antigenic identity. After growth in a chemically defined medium, stage I (lyophilized) culture supernatants were assayed for activity with *N*-acetylneuramin lactose, human α-1-acid glycoprotein, fetuin, colominic acid, and bovine submaxillary mucin. Neuraminidase produced by *P. multocida* A:3 was purified by a combination of salt fractionation, ion-exchange chromatography on DEAE-Sephacel, and gel filtration on Sephadex G-200. Purified *P. multocida* A:3 neuraminidase was employed to immunize rabbits, and the resulting antiserum reduced the activity of the *P. multocida* A:3 enzyme by 40.3%. This antiserum also reduced the activities of the neuraminidases produced by other serotypes by between 30.8 and 59.6%. Molecular weight estimates of the neuraminidases produced by the various serotypes were obtained by gel filtration chromatography on Sephadex G-200. Each of the 16 serotypes examined produced a neuraminidase with a molecular weight of approximately 500,000. In addition, all 16 high-molecular-weight neuraminidases showed similar substrate specificities. On the basis of these data, it appears that the high-molecular-weight neuraminidases produced by different *P. multocida* serotypes are quite similar.**

*Pasteurella multocida* has been shown to be an important cause of the animal disease syndrome known as pasteurellosis. *P. multocida* diseases include pneumonia in sheep, pigs, and cattle and acute septicemia in turkeys and chickens (4). There are 16 known somatic serotypes of *P. multocida* (numbered 1 through 16), and these serotypes can occur in any of five separate capsular groups (A, B, D, E, or F [27]). The somatic serotypes are determined by a gel diffusion precipitin test, which employs heat-stable antigens extracted from a formalinized saline bacterial suspension (20). Capsular type A *P. multocida* strains produce snuffles in rabbits, cholera in fowl, and pneumonia in sheep, cattle, and pigs (4, 14). *P. multocida* capsular types B and E have been demonstrated to cause hemorrhagic septicemia in cattle and buffaloes (4). Type D *P. multocida* strains have been shown to produce pneumonia in cattle and atrophic rhinitis in swine (4, 21).

In light of the fact (5) that *P. multocida* serotype A is occasionally responsible for a severe fibrinous pleuropneumonia in cattle and sheep (more commonly referred to as shipping fever), we have attempted to examine some of the bacterial exoproducts that could be involved in pneumonic tissue pathology. Most recently, our studies have examined the neuraminidase produced by *P. multocida* (26). Scharmann et al. were the first to report neuraminidase production by *P. multocida* (22). These workers demonstrated that 102 of 104 *P. multocida* strains produced this enzyme. Later, Drzeniek et al. showed that almost all *P. multocida* strains they examined produced a sialidase (7). Recently, Ifeanyi and Baillie (11) demonstrated passive protection of mice with antiserum to neuraminidase from *P. multocida* A:3. That study indicated that the virulence of *P. multocida* may depend, in some way, on the production of its neuraminidase.

Neuraminidase production has been implicated as a virulence factor in bacteria such as *Vibrio cholerae* (19), *Streptococcus pneumoniae* (12), and *Corynebacterium diphtheriae* (15). Although it is not known how bacterial neuraminidase acts as a virulence factor, one mechanism was proposed by Gottschalk (9). He demonstrated that by removing sialic acid from salivary glycoproteins, the protective capabilities of these secretions against potential pathogens were inhibited. In this scenario, the bacterial extracellular neuraminidase would allow the organism to survive this in vivo defense mechanism.

Numerous papers which describe neuraminidase production by *P. multocida* have previously been published (7, 11, 16–18, 22, 26). However, only two papers have dealt with characterization of this enzyme (7, 26). Both reports examined capsular group A *P. multocida* strains. In one of these papers (26), the *P. multocida* A:3 neuraminidase was extensively characterized. We therefore thought it would be of importance to further examine the characteristics of neuraminidase production by the other 15 *P. multocida* serotypes (serotypes 1, 2, and 4 through 16). In this study, we examined the molecular weight, substrate specificity, and antigenic structure of this enzyme produced by various *P. multocida* serotypes.

**Bacterial strains, media, and growth conditions.** Sixteen strains of *P. multocida* (serotypes 1 through 16) were kindly supplied by R. B. Rimler and were used in this study. These strains have been previously described (20) and are listed in Table 1. Cultures were stored at −85°C in reconstituted double-strength powdered milk on filter paper. For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on nutrient agar (Difco, Detroit, Mich.) plus 5% bovine erythrocytes. Frozen stock cultures were transferred only once before being used in experiments. Colonies from blood agar were used to initiate cultures in defined medium. The medium and growth conditions in RPMI 1640 buffered with 25 mM HEPES

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TABLE 1. Capsule groups, somatic serotypes, and host sources of *P. multocida* strains employed[a]

| Strain | Capsule group[b]: somatic serotype | Host |
|---|---|---|
| X-73 | A:1 | Chicken |
| M-1404 | B:2 | Bison |
| P-1059 | A:3 | Turkey |
| *P. multocida* A:3[c] | A:3 | Bovine |
| P-1662 | A:4 | Turkey |
| P-1702 | A:5 | Turkey |
| P-2191 | —:6 | Turkey |
| P-1997 | —:7 | Herring gull |
| P-1581 | —:8 | Pine siskin |
| P-2095 | A:9 | Turkey |
| P-2100 | A:10 | Turkey |
| P-903 | —:11 | Swine |
| P-1573 | A:12 | Human |
| P-1591 | —:13 | Human |
| P-2225 | A:14 | Bovine |
| P-2237 | —:15 | Turkey |
| P-2723 | A:16 | Turkey |

[a] Abbreviated from reference 20.
[b] The absence of a letter (—) signifies a nonencapsulated strain.
[c] Isolated from a confirmed case of acute bovine respiratory disease (26).

(*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Gibco, Grand Island, N.Y.) have been previously described (23).

**Purification of the extracellular neuraminidase produced by *P. multocida* A:3 and molecular weight estimates of the neuraminidases produced by various *P. multocida* serotypes.** The extracellular neuraminidase of *P. multocida* A:3 was purified by the procedure of White et al. (26), including lyophilization (stage I), 100% ammonium sulfate fractionation (stage II), ion-exchange chromatography on DEAE-Sephacel (stage III), and gel filtration chromatography on Sephadex G-200 (stage IV). Gel filtration chromatography on a Sephadex G-200 column (2.5 by 90 cm; Pharmacia Fine Chemicals) was performed to determine the molecular weights of the neuraminidases produced by serotypes 1 to 16. The elution volumes were determined by recording the volume at which the following proteins displayed a maximal $A_{280}$: blue dextran, 2,000,000; thyroglobulin, 669,000; sweet potato β-amylase, 200,000; alcohol dehydrogenase, 150,000; ovalbumin, 43,000; and RNase, 13,700. Partition coefficients were then determined for the eluted peaks of neuraminidase activity. The molecular weight of each neuraminidase was estimated by interpolation from a graph of partition coefficient versus $\log_{10}$ molecular weight (25).

**Assays.** The amounts of neuraminidase activity present in concentrated cultural supernatant fluids or purified preparations were quantitated by measuring the amount of sialic acid released from five substrates as previously described (25). The five substrates employed, with known α-ketosidic linkages, included *N*-acetylneuramin lactose, colominic acid, fetuin, α-1-acid glycoprotein, and bovine submaxillary mucin (Sigma Chemical Co.). Protein determinations were performed in duplicate by the method of Lowry et al. (13), with bovine serum albumin (Sigma) as the standard.

**Neutralization of heterologous *P. multocida* neuraminidases with antiserum to *P. multocida* A:3 neuraminidase.** The preparation of this antiserum in rabbits has previously been described (26). Rabbit serum was separated from clotted blood by a method reported earlier (3). The presence of neutralizing antibody to high-molecular-weight neuraminidases of all 16 serotypes was determined by neuraminidase assay (25). The various stage I preparations (100 μl) were incubated with either 0.1 ml of preimmune serum or 0.1 ml of immune serum in the presence of 0.1 mg of fetuin, 10 mM $CaCl_2$, and 33.3 mM sodium acetate (pH 6.0) in a final volume of 0.4 ml. Each set of assays included a substrate blank, and enzyme reactions were initiated by the addition of the enzyme to the remaining components. The enzyme and serum preparations were incubated together for 90 min at 4°C on ice before the assay was begun to allow the antibody to react with the enzyme. After incubation, the percent reduction in neuraminidase activity was determined by performing the neuraminidase assay (25) for 60 min at 37°C and comparing the differences in activities between the preimmune and immune sera. Statistical evaluations were performed by employing Student's *t* test in a pairwise comparison.

**Substrate specificities of neuraminidases produced by strains of different *P. multocida* serotypes.** The substrate specificity of the stage IV neuraminidase produced by *P. multocida* A:3 was previously described (26). This enzyme was active against all substrates utilized in these studies. This was also the case for the stage I neuraminidase preparation from a different *P. multocida* A:3 strain (P-1059). Table 2 shows the release of sialic acid from various substrates by neuraminidases from 16 serotypes of *P. multocida*. All 16 serotypes appeared to produce neuraminidase. The majority of these enzymes were most active against *N*-acetylneuramin lactose, fetuin, and α-1-acid glycoprotein, although there were exceptions. Bovine submaxillary mucin was the substrate least utilized by *P. multocida* neuraminidases. The *P. multocida* neuraminidases from serotypes 11, 12, 15, and 16 were not active against this substrate.

**Molecular weight estimations of neuraminidases produced by all *P. multocida* serotypes.** Stage I neuraminidases from all *P. multocida* serotypes were chromatographed on Sephadex G-200 to purify the enzymes and obtain molecular weight estimates. All 16 serotypes produced high-molecular-weight

TABLE 2. Release of sialic acid from various substrates by neuraminidases from 16 serotypes of *P. multocida*[a]

| Substrate[b] | Sialic acid released (μmol/min/mg of protein) by serotype[c] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| *N*-Acetylneuramin lactose | +++ | ++ | ++ | ++ | +++ | +++ | ++ | ++ | +++ | +++ | ++ | ++ | +++ | ++ | +++ | +++ |
| Fetuin | +++ | ++ | ++ | ++ | +++ | ++ | ++ | ++ | ++ | ++ | + | ++ | +++ | ++ | ++ | +++ |
| α-1-Acid glycoprotein | +++ | ++ | ++ | ++ | +++ | ++ | +++ | ++ | + | ++ | + | + | ++ | ++ | +++ | +++ |
| Colominic acid | ++ | ++ | ++ | ++ | ++ | +++ | ++ | ++ | ++ | +++ | + | + | +++ | ++ | +++ | ++ |
| Bovine submaxillary mucin | + | ++ | + | + | + | + | + | ++ | + | + | − | − | + | + | − | − |

[a] Stage I neuraminidases were prepared as previously described (26).
[b] The concentration of each substrate was 1 mg/ml.
[c] Data are averages of two determinations. −, activity of less than 2 nmol; +, activity of between 2 and 50 nmol; ++, activity of between 51 and 100 nmol; +++, activity of greater than 100 nmol.

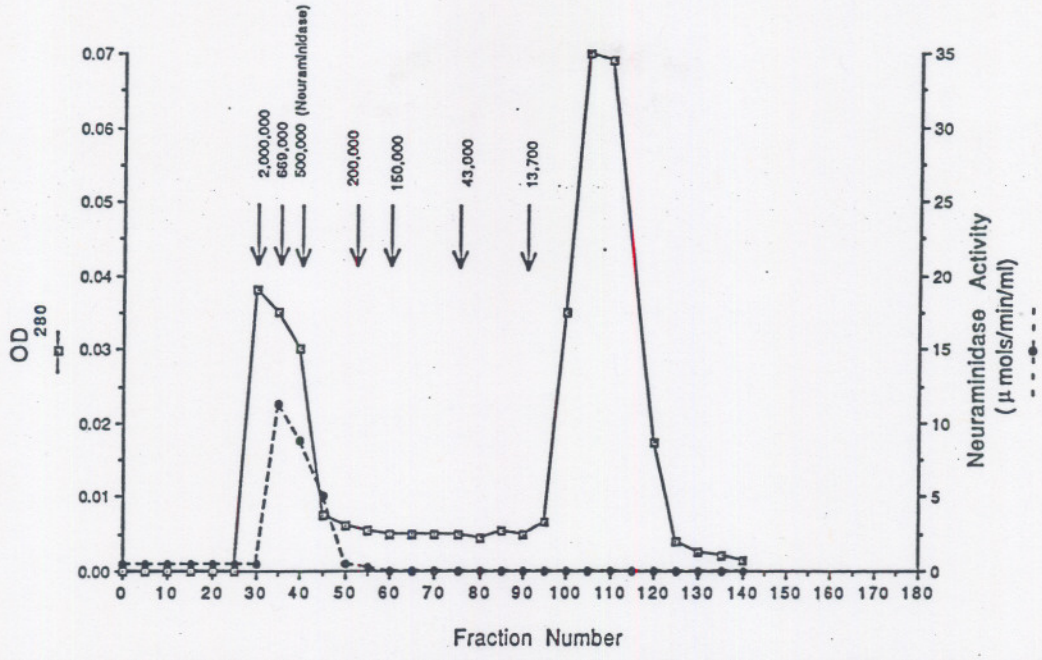


FIG. 1. Sephadex G-200 elution profile of stage I neuraminidase from *P. multocida* serotype 16. Stage I enzyme was applied to the column and eluted with 10 mM sodium citrate buffer (pH 6.0) at 4°C. Every fifth tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration of the column with blue dextran and proteins of known molecular weights was performed with the same buffer.

neuraminidases of approximately the same molecular weight. These neuraminidases had an apparent molecular weight of approximately 500,000 by chromatography on Sephadex G-200. Figure 1 illustrates the elution profile of stage I neuraminidase from *P. multocida* serotype 16 on Sephadex G-200.

**Neutralization of neuraminidases produced by heterologous *P. multocida* serotypes with anti-*P. multocida* A:3 neuraminidase.** In the presence of immune serum, a 40.3% reduction in activity of the homologous stage I *P. multocida* A:3 enzyme preparation was observed (Table 3). This was shown to be statistically significant at a $P$ value of 0.0119. The stage I heterologous neuraminidase preparations obtained from other serotypes were also incubated with either preimmune or immune serum against stage IV *P. multocida* A:3 neuraminidase and then assayed for activity. In the presence of immune serum, the percent reduction in neuraminidase activity of stage I preparations obtained from heterologous serotypes (Table 3) ranged from 30.8% (serotype 7) to 59.6% (serotype 8). The percent reductions in neuraminidase activity between the preimmune and immune serum incubations were relatively similar among strains of different serotypes. These reductions were all shown to be statistically significant (except for serotype 3), with $P$ values of $\leq 0.05$. There were no significant differences in percent reduction of neuraminidase activity between serotypes.

We recently characterized the production of *P. multocida* A:3 neuraminidase by a strain isolated from a case of acute bovine pneumonia (26). In that study, a method of producing neuraminidase was developed for *P. multocida* A:3 strains. Conditions which are optimal for the production of *P. multocida* A:3 neuraminidase may not be suitable for producing neuraminidases from other *P. multocida* serotypes and capsule groups. For this reason, we examined the relationship between the *P. multocida* A:3 neuraminidase and the neuraminidases produced by other capsule groups and serotypes.

The first characteristic of neuraminidase that we examined was substrate specificity. Neuraminidase produced by *P. mul-*

TABLE 3. Antibody neutralization by anti-*P. multocida* A:3 neuraminidase of neuraminidases produced by heterologous serotypes of *P. multocida*[a]

| Serotype | % Reduction in activity[b] |
|---|---|
| 1 | 36.3[c] |
| 2 | 46.4[c] |
| 3 | 35.9 |
| 3[d] | 40.3[c] |
| 4 | 36.9[c] |
| 5 | 44.0[c] |
| 6 | 54.0[c] |
| 7 | 30.8[c] |
| 8 | 59.6[c] |
| 9 | 33.6[c] |
| 10 | 32.2[c] |
| 11 | 41.5[c] |
| 12 | 43.4[c] |
| 13 | 44.1[c] |
| 14 | 35.3[c] |
| 15 | 51.5[c] |
| 16 | 53.7[c] |

[a] Stage I neuraminidase activity after incubation with serum. Either preimmune or immune serum was incubated with stage I enzyme from each *P. multocida* serotype, and the activity against fetuin was assayed. Data are the means of eight determinations.

[b] The percent reduction in neuraminidase activity was obtained by comparing neuraminidase activities after a 90-min preincubation on ice and a 60-min incubation at 37°C in the presence of preimmune and immune sera and dividing the difference by the preimmune value.

[c] Statistically different from preimmune serum postincubation activity against the appropriate *P. multocida* serotype neuraminidase ($P \leq 0.05$), as determined by Student's $t$ test in a pairwise comparison.

[d] Purified neuraminidase from this organism was employed to produce the anti-neuraminidase in rabbits, as previously described (26).

[e] Statistically different from preimmune serum postincubation activity against *P. multocida* A:3 neuraminidase ($P = 0.0119$), as determined by Student's $t$ test in a pairwise comparison.

*tocida* A:3 was found to be active against all compounds of known α-ketosidic linkages examined (Table 2). These results agree with those obtained in a previous study (26). In that study, the *P. multocida* A:3 neuraminidase was most active against *N*-acetylneuramin lactose, followed in decreasing order of activity by human α-1-acid glycoprotein, fetuin, colominic acid, and bovine serum albumin. This was essentially the same pattern for the other 15 serotypes of *P. multocida*, with a few exceptions. A broad range of substrate specificity by a bacterial neuraminidase has been observed before. For example, *V. cholerae* (6), *S. pneumoniae* (10), an *Arthrobacter* species (8), and *Clostridium perfringens* (6) have been shown to produce neuraminidases which cleave sialic acid from more than one substrate.

We next examined the molecular weights of the neuraminidases produced by various *P. multocida* serotypes. The molecular weight for the *P. multocida* A:3 neuraminidase was demonstrated by several groups to be between 250,000 and 500,000. In 1972, Drzeniek et al. (7) reported that a neuraminidase was produced by nearly all *P. multocida* strains examined, and those authors partially purified the enzyme. They reported that the enzyme had a molecular weight of 250,000. White et al. (26) reported that the *P. multocida* A:3 neuraminidase had an apparent molecular weight of approximately 500,000 by Sephadex G-200 column chromatography and a range of 345,000 to 420,000 when sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used. This was confirmed in the present study. Each *P. multocida* neuraminidase had a molecular weight of approximately 500,000 when Sephadex G-200 chromatography was used. This indicates that the neuraminidases of *P. multocida* have molecular weights greater than those of the neuraminidases of other organisms described to date. For example, the molecular weights of the neuraminidases of *C. perfringens*, *V. cholerae*, and *S. pneumoniae* are 61,000, 68,000, and 81,000, respectively (2). The only other bacterial neuraminidase with a molecular weight comparable to that of *P. multocida* neuraminidase is the extracellular neuraminidase of *Pasteurella haemolytica*, which is reported to be between 150,000 and 200,000 (23, 25).

Purified neuraminidase (stage IV from *P. multocida* A:3) was used to immunize New Zealand White rabbits to produce antibody specific for the enzyme produced by this strain. The presence of anti-neuraminidase antibody was detected by the standard neuraminidase assay and was based on the reduction of thiobarbituric-acid-reactive material after a 90-min incubation of the enzyme-antibody mixture at 4°C (Table 3). The results indicate that the neuraminidases produced by different serotypes of *P. multocida* are antigenically similar. It appears that similar epitopes make up the enzymatically active portion of all *P. multocida* neuraminidases.

In this study, we demonstrated that the neuraminidases produced by various serotypes of *P. multocida* are neutralized by specific anti-*P. multocida* A:3 neuraminidase antibody and are similar in molecular weight and substrate specificity. Thus, workers in this area can study these enzymes by using the characterization scheme previously developed for the neuraminidase produced by *P. multocida* A:3 (26). The contribution or lack thereof of neuraminidase production by *Pasteurella* species to the virulence of these bacteria remains unknown. We have recently demonstrated that *P. haemolytica* serotypes 2 and 6 produce more neuraminidase in vitro than do *P. haemolytica* serotype 1 and *P. multocida* serotypes (24). However, the significance of these results awaits further clarification.

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